Amendments to the Specification

On Page 1, just below the title, please add the following new paragraph:

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of US Non-provisional Patent Application No. 09/738,961, filed December 15, 2000, which claims priority to US Provisional Patent Application No. 60/170,983, filed December 15, 1999.

On Page 1, just below the first paragraph having the heading "CROSS-REFERENCE TO RELATED APPLICATIONS," please add the following heading:

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

On Page 1, please replace the paragraph following the heading "BACKGROUND" with the following amended paragraph:

Since the concept of protein or drug delivery from polymers was first introduced, research efforts have focused on developing polymer formulations that would be widely applicable for delivery of biologically active agents, such as proteins, peptides, oligonucleotides, DNA, low molecular weight drugs and vaccine antigens. Efforts to this end have intensified recently since hundreds of recombinant proteins and other biotechnological [[drgs]] drugs and vaccine antigens are in the pipeline for FDA approval, and the current[[,]] method of protein delivery generally requires injections on a daily basis. Frequent dosing is clinically undesirable due to patient discomfort, psychological distress, and poor compliance for administering self-injections. To reduce injection frequency, peptide and protein drugs are encapsulated in biodegradable polymers, which are processed into a form that is easily administered through a syringe needle. Current preparations on the market for the delivery of small peptides can reduce the frequency of injections to once every 1-3 months depending on the size and dose of the polymer implant. This incubation time, for which a large globular protein must remain encapsulated in the polymer at physiological temperature, poses significant challenges to retain both the structural integrity and the biological activity of the protein.

Please replace the last paragraph on page 4, starting at line 20, with the following amended paragraph:

Stabilization of the encapsulated agent is achieved by providing a delivery system whose microclimate, i.e. the pores where the active agent resides, uniformly or homogeneously maintain a pH of greater than 3 and less than 8, preferably greater than 4 and less than 8, more preferably from 5 to 7.5 during biodegradation. To determine if the method has provided a ~~plymeric~~ polymeric delivery system whose microclimate homogenously maintains a pH of between 3 and 8, 1% w/w BSA is dispersed in the polymer solution during manufacture by the chosen method and the extent of ~~aggreagation~~ aggregation of this protein is assayed after 4 weeks of incubation of the polymeric delivery system in phosphate buffered saline with 0.02% Tween 80 at 37° C. If the amount of residual BSA that has formed water insoluble noncovalent

aggregates (i.e., soluble in 6 M guanidine hydrochloride or 6 M urea) is less than or equal to 15% of the total BSA in the prepared polymer dosage form, the method has produce a polymeric delivery system whose microclimate homogeneously maintains a pH of between 3 and 8.

Please replace the first full paragraph on page 8, starting at line 4, with the following amended paragraph:

Controlled-release systems for proteins and peptides using poly(lactide-*co*-glycolide) (PLGA) have been studied for more than one decade. Although this type of biodegradable polymer has been successful in delivery of small peptides such as LHRH analogues [1], the delivery of large globular proteins in PLGA has been limited because of the irreversible inactivation of these therapeutic agents prior to their release *in vivo* [[2, 3]]. Previous work from our group has shown that encapsulated bovine serum albumin (BSA) in PLGA systems forms insoluble non-covalent aggregates and is hydrolyzed after incubation in a physiological buffer at 37°C for 28 days. The acidic pH and intermediate water content existing in the polymer were implicated as two major factors causing instability of the encapsulated protein, and the BSA was stabilized by co-encapsulating poorly water-soluble basic inorganic salts such as $Mg(OH)_2$. The incorporation of the basic additive in the formulation was also successful in stabilizing therapeutic proteins such as recombinant human basic fibroblast growth factor and bone morphogenetic protein-2.

Please replace the second full paragraph on page 8, starting at line 17, with the following amended paragraph:

In this study, to further characterize the stabilization mechanism by co-encapsulation of $Mg(OH)_2$, the effect of basic additive type and content on protein stability and release kinetics in PLGA delivery devices was studied. Since acid-induced inactivation pathways (e.g., at pH < 3) are common for most proteins, BSA was selected as a model protein. BSA undergoes unfolding from its F to E form at pH 2.7, and forms non-covalent aggregates in PLGA presumably due to this unfolding [[4]]. The influence of $Mg(OH)_2$ on the delivery system such as pH change in the release medium, polymer degradation and water uptake kinetics was also examined. In addition, the basicity of the salt as well as the loading of base and protein [[was]] <u>were</u> examined for their effects on BSA aggregation.

Please replace the first full paragraph on page 9, starting at line 3, with the following amended paragraph:

Poly(DL-lactide-*co*-glycolide) ~~50150~~ <u>50/50</u> with inherent viscosity of 0.23, 0.41, and 0.63 dl/g in hexafluoroisopropanol were purchased from ~~Birmin ham~~ <u>Birmingham</u> Polymers, Inc. (Birmingham, AL). Bovine serum albumin (A-3059, Lot 32HO463) was purchased from Sigma Chemical Co. (St. Louis, MO). Poly(vinyl alcohol) (80% hydrolyzed with Mw range of 8,000-9,000), $Mg(OH)_2$, $Ca(OH)_2$, and $Ca_3(PO_4)_2$ were obtained from Aldrich Chemical Co. (Milwaukee, WI). $ZnCO_3$ was from ICN Biopharmaceuticals Inc. (Aurora, OH). All these salts were fine powders (< 5 μm) and were used as received.

Please replace the second full paragraph on page 9, starting at line 11, with the following amended paragraph:

A solvent extrusion method similar to that used previously by [[Our]] our group for intraocular implants [[[51]]] was used to prepare the PLGA cylinders with a diameter on the millimeter scale, which we term *millicylinders.* Briefly, a uniform suspension of sieved protein powder (< 90 μm) with or without basic salt in 50% (w/w) acetone-PLGA 50/50 solution was loaded in a syringe and extruded into a silicone tubing (I.D. 0.8 mm) at about 0.1 ml/min. The solvent extruded suspension was dried at room temperature for 24 h and then dried in a vacuum oven at 45°C for another 24 h before testing. The protein loading was calculated as the percentage of amount of BSA versus the total weight of mixture (i.e., protein, polymer, and salt).

Please replace the connecting paragraph on pages 9-10, starting at page 9, line 27, with the following amended paragraph:

Protein stability was assessed by the percentage of water insoluble non-covalent BSA aggregates generated within the implants versus the initial encapsulated protein. Protein stability within PLGA implants was analyzed as follows: First, millicylinders with a length of [I] 1 cm were incubated under 80% and 96% relative humidity (RH) at 37°C for 21 days. Then, the polymer was dissolved in acetone and centrifuged to spin down the protein. The remaining protein pellet was washed three times with acetone and then air-dried. The final protein pellet was analyzed as in *Analysis of the Protein ~~Exactedjrom~~ Extracted from PLGA Implants.* The protein remaining in PLGA implants after release in PBST at 37°C for 28 days was also extracted similarly and analyzed as above.

Please replace the first full paragraph on page 10, starting at line 6, with the following amended paragraph:

The BSA pellet extracted from PLGA implants was first reconstituted in PBST and incubated at 37°C overnight to determine the soluble protein fraction remaining in the polymer. Any remaining aggregates were collected by centrifugation again, and brought up in the denaturing solvent (PBST/6 M urea/1 mM EDTA) and incubated at 37°C for 30 min to dissolve ~~non-coval!ent~~ non-covalent bonded BSA aggregates. Then, any final undissolved BSA aggregates were collected again and dissolved in the reducing solvent (the denaturing solvent plus 10 mM DTT) to dissolve any disulfide-bonded aggregates [[[7]]].

Please replace the last paragraph on page 10, starting at line 28, with the following amended paragraph:

Weight-averaged molecular weight ([[M,]] M_w) of the degraded polymers was measured by gel permeation chromatography (GPQ on a Styragel™ HR 5E column (7.8 x 300 mm, Waters, Milford, MA), which was performed on a HPLC system (Waters, Milford, MA) equipped with a refractive index detector (Hewlett Packard). The mobile phase was tetrahydrofuran with a flow rate of 1 ml/min. Mw was calculated based on polystyrene standards (Polysciences Inc., PA) using Millenium Software Version 2.10.

Please replace the fourth full paragraph on page 11, starting at line 18, with the following amended paragraph:

Earlier work demonstrated that after an initial burst on the first day, BSA release from 15% BSA/millicylinders (0.63 dl/g PLGA 50/50) during 4 weeks incubation in PBST at 37°C is insignificant[[.]], and the remaining protein mostly becomes water-insoluble non-covalent aggregates. It has been shown that the BSA aggregation is mainly caused by acidic microclimate pH generated by polymer degradation and water uptake by the polymer during incubation in PBST. It has also been found that incorporation of 3% $Mg(OH)_2$ into 15%BSA/PLGA50/50 millicylinders can increase BSA release from PLGA cylindrical implants and reduce BSA aggregation. Structural characterizations by using SDS-PAGE, IEF, CD, and fluorescence spectroscopy have confirmed that the structure of BSA from 3%$Mg(OH)_2$/15%BSA devices is mostly retained in a native form.

Please replace the fifth paragraph on page 11, starting at line 28, with the following amended paragraph:

To examine the effect of ~~Mg(OH)2~~ $Mg(OH)_2$ content, the base was co-encapsulated in 15%BSA/PLGA millicylinders as a function of base loading and the BSA release. The study was carried out in PBST at 37°C. With the increasing $Mg(OH)_2$ content from 0.5% to 6%, both BSA release rate and total releasable amount of protein increased. The residual BSA remaining in these devices after the 4-week release interval was analyzed. In the absence of $Mg(OH)_2$, most of the remaining protein became water insoluble aggregates, which were nearly completely soluble in the denaturing solvent (i.e., non-covalent aggregates were formed). As the content of $Mg(OH)_2$ was increased, the amount of water-insoluble aggregates decreased. As $Mg(OH)_2$ content was raised to 6%, almost no aggregates were formed within the device. For all the aggregates, an insignificant amount of covalent aggregates was observed in each polymer specimen. These results indicate that an increase in $Mg(OH)_2$ content even up to 6% does not generate an alkaline microclimate in the polymer during release.

Please replace the first full paragraph on page 34, starting at line 2, with the following amended paragraph:

When a small amount of bFGF was encapsulated to the $Mg(OH)_2$/BSA/PLGA millicylinders (~0.0025%), the growth factor was released ~~(filled circles in Fig. 4A)~~ in a fashion similar to that observed for BSA ~~(Fig. 1)~~. Over 28 days, 71% of bFGF was detected by ELISA in the release medium and 21% remained in the polymer fraction (Table 3). This accounts for about ~92% of the initially encapsulated bFGF. It is important to note that when the millicylinders did not contain both heparin and the $Mg(OH)_2$/BSA combination, bFGF lost immunoreactivity. For example, if heparin was removed from the stable formulation, only 2% bFGF was released over one month with no immunoreactive bFGF in the residual fraction (Table 3). Similarly, if 20% arabic gum was substituted for 3% $Mg(OH)_2$/15% BSA (a 0%$Mg(OH)_2$/15%BSA control could not be performed because of BSA aggregation), no bFGF was observed in the release medium after 4 days and only 38% was accounted for in both the release and residual fraction.

Please replace the second full paragraph on page 34, starting at line 14, with the following amended paragraph:

To increase the capacity of the polymer to deliver bFGF, we increased the bFGF loading to 0.01% and the sucrose loading to 21.3 %. ~~As seen in Fig. 4A *(filled squares),* the~~ The bFGF release initially is much slower and later exhibits a linear release profile up to 4 weeks. The release of BSA from the same preparation in PBST was similarly retarded ~~*(data not shown)*~~. This indicates that sucrose can be used to slow down the release of both BSA and bFGF from the polymer, probably by increasing the viscosity of the aqueous pores in the polymer.

Please replace the third full paragraph on page 34, starting at line 20, with the following amended paragraph:

The release kinetics of bFGF ~~in Fig. 4A~~ demonstrated that soluble bFGF is released continuously with BSA. However, immunoreactive bFGF does not guarantee bioactivity. To test the bioactivity of bFGF released from PLGA, we examined the bFGF samples in the release and residual fraction by the ability of the growth factor to induce cell proliferation (as indicated by ^{3}H-thymidine incorporation (20)). ~~As shown in Fig. 4B (time 0), the~~ The encapsulation procedure did not affect the biological activity of bFGF. Some small inactivation apparently occurred during the release experiment, but 65-85% of bFGF was bioactive over the entire release interval, confirming that the majority of immunoreactive bFGF was biolooically active. Thus, by neutralizing the acidic microclimate in PLGA, we have prepared an injectable PLGA device that delivers bioactive bFGF for more than one month.

Please replace the first full paragraph on page 36, starting at line 8, with the following amended paragraph:

We note that it has been suggested that BSA becomes unstable in PLGA microspheres primarily by protein adsorption to the polymer (44). This conclusion was strongly weighed on the ability of SIDS to cause liberation of previously unreleasable BSA from the polymer. We remark that the SDS buffer we used in the SDS-PAGE ~~in Fig. 2B~~ dissolves the noncovalent aggregates formed in the polymer. This solubilization effect may explain the reported release of sequestered BSA from the polymer caused by the surfactant. Therefore, **[[**vie**]]** we conclude that protein adsorption, consistent with our simulations described earlier**[[-]]**, is not the predominant source of instability of BSA in PLGA microspheres.

Please replace the first paragraph under the heading "Microsphere composition and phase behavior analysis" on page 41, starting on line 7, with the following amended paragraph:

~~In Fig. 1, the~~ The IR spectra of blank microspheres prepared from pure PEG, pure PLA (i.v. = 1.07 dl/g), and a PLA/PEG ~~blend are~~ displayed **[[**. A**]]** a broad band at 2876 cm^{-1} and a peak at 1757 cm^{-1}, which were assigned to the CH_2 stretching on the PEG unit and the carbonyl group (C-O) of PLA, respectively. Both characteristic peaks for CH_2 and C=O appeared in the IR spectrum of the blend of PLA and PEG. By estimating the PEG content in the blend with a calibration curve generated from PLA and PEG physical mixtures with different weight ratios, complete incorporation of PEG in PLA matrix by the O/O encapsulation method was indicated ~~*(data not shown)*~~.

Please replace the first paragraph under the heading "Microsphere morphology" on page 41, starting on line 21, with the following amended paragraph:

~~As seen in Fig. 2, after~~ After preparation, microspheres with different weight ratios of PLA and PEG had spherical and smooth surfaces. An average size of ~100 μm was recorded for these microsphere preparations. After 35 days of incubation, microspheres prepared from 100% PLA remained intact with a smooth surface. With the blend of PEG, the microsphere structure still remained intact, but a small amount of pores appeared on the PLA/PEG microspheres surface. With higher PEG blend, more pores became visible. In addition;, the microsphere surface showed indentations, which may have occurred during drying of the particles before analysis, The SEM images suggested that the incorporation of PEG into PLA created more channels in the microspheres, which may have increased the permeability to the encapsulated protein. In addition, the microsphere surface likely consisted of a PLA-rich phase, whereas the interior of microspheres was likely PEG-rich. Otherwise, more pores created by PEG solubilization would be expected on the rnicrosphere surface. The PLA-rich surface phenomenon is possibly due to the higher hydrophobicity and longer chain of PLA, which could have caused selective PLA precipitation at the surface during the O/O microsphere preparation. Further surface analysis would be required for a definitive conclusion.

Please replace the first paragraph after the heading "Release kinetics and stability of BSA in the PLA/PEG microspheres" on page 42, starting at line 6, with the following amended paragraph:

To investigate the effect of PEG in the PLA/PEG microspheres, microspheres with different weight ratios of PEG 10,000 to PLA were prepared and the BSA controlled release was monitored in PBST at 37°C. Theoretical BSA loading of all these formulations was 5% and encapsulation efficiency vas invariably between 90% and 100%. ~~As seen in Fig. 3A, when~~ When PEG content was less than 10% of polymer weight, similar release kinetics of BSA from microspheres was observed and less than 45% of BSA was released after a 4-week incubation. When PEG content was raised to 20%, the total releasable amount of protein was significantly increased to 75%. In addition, the effect of PEG molecular weight on protein release was also evaluated. ~~As seen in Fig. 3B,~~ BSA had almost identical release kinetics in microspheres irrespective of whether PEG 10,000 and PEG 35,000 was used (the weight ratio of PEG/PLA was 20:80). When PEG 35,000 content was increased to 30% in PLA/PEG microspheres, a higher burst release of BSA was observed.

Please replace the paragraph that starts on page 42, line 29 and carrying-over to page 43 with the following amended paragraph:

The integrity of the soluble BSA recovered from the polymer (28-day incubation) was further examined by SDS-PAGE. ~~As seen in Fig. 4A, some~~ Some peptide fragments were observed in lanes 6 and 7 (formulations *a* and *b*), indicating mild peptide bond hydrolysis occurred during incubation. In contrast, soluble BSA recovered from formulations containing more than 20% PEG showed a very similar band with standard BSA and no degradation product bands were .noticeable. Soluble BSA recovered from formulations *c*, *d* and *e* was further examined by IEF. No pI alterations in BSA were observed in these samples. Likewise, secondary

and tertiary structure of BSA was similar to standard BSA control. Hence, the structure of BSA in formulations *c*, *d*, and *e* was retained within the polymer for one month.

Please replace the second full paragraph under the heading "Mechanisms of BSA stabilization in the PLA/PEG microspheres" on page 43, starting at line 9, with the following amended paragraph:

To address this question, we first examined the pH change of the release medium when the PLA/PEG formulations were incubated at 37°C and PEST (pH 7.4). Unlike PLGA 50/50, which showed a dramatic pH drop in the release medium after 4-week incubation (3), both PLA and PLA/PEG formulations remained a relatively neutral pH (above 7) in the release medium over 29 days of incubation ~~(Fig. 5)~~. However, a slightly lower pH in the release medium incubated with PLA/PEG formulation than that in PLA was observed (-0.1 -0.2 pH units difference). This result suggested that some acidic degradation products were able to diffuse out of polymer device through the water channels formed by PEG in PLA/PEG formulation. In addition, by using a previously reported method (pH determination of polymer solution in the mixture of ACN and water) (12.), the pa_H* inside formulation *d* before and after 30-day incubation was determined as 6.5 and 5.4, respectively ~~*(data not shown)*~~, suggesting a very small accumulation of acid in the polymer. In contrast, PLGA 50/50 microspheres were reported to reach pa_H* ~3 after similar incubation time (5). These results demonstrated that the acid build[[.]]-up was largely reduced in the PLA/PEG blend formulation.

Please replace the paragraph that starts on page 43, line 29 and carrying-over to page 44 with the following amended paragraph:

The water content difference in formulations during release was compared by performing a water uptake kinetics study of microspheres at 97% relative humidity [[(RH]]]. Under controlled humidity, microspheres will adsorb water vapor and potential water uptake of different formulations during release can be predicted and compared. ~~As seen in Fig. 6, PEG35,000~~ PEG 35,000 showed a strong water uptake. On the second day, the water content in ~~PEGE5,000~~ PEG 35,000 blank microspheres was almost 120% of the dry microsphere weight. Upon blending PEG in the formulation, the water uptake rate was significantly increased. The higher the PEG content, the higher the increase in water uptake. Microspheres containing 20% PEG had almost twice the amount of water uptake; relative to those with 10% of PEG in the humid environment[[,]]. When microspheres are incubated in the release medium, higher water content in the PLA/PEG blend is expected. The presence of 5% BSA did not increase water uptake rate significantly in the blend. formulation. The water uptake in the blend was likely overwhelmed by the strong water ~~sorption~~ adsorption by PEG.

Please replace the second full paragraph on page 45, starting at line 12, with the following amended paragraph:

PEG is hydrophobic in nature and it may potentially interact with the hydrophobic groups of BSA and induce BSA unfolding. It was reported that [[PEC,t]] PEG of low Mw 1000 and 4000 interacts favorably with hydrophobic sides chains of human serum albumin (hSA), leading to a stabilization of the unfolded state (15). To test the interaction of high Mw PEG with BSA, GnCl unfolding curve of BSA with the addition of PEG 10,000 and PEG 35,000 (the weight ratio

of BSA to PEG was 1:5) was determined by fluorescence spectroscopy. ~~As seen in Fig. 6, similar~~ Similar unfolding curves were observed in three preparations, The conformational stability of BSA was therefore likely not affected by the addition of PEG 10,000 and PEG 35,000 with 1:5 ratio of BSA to PEG.